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A two-stage, two-organism process for biohydrogen from glucose

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Abstract
H2 can potentially be produced in a two-stage biological process: the fermentation of glucose by *Escherichia coli* HD701 and the photofermentation of the residual medium by *Rhodobacter sphaeroides* O.U. 001. In a typical batch fermentation, *E. coli* consumed glucose and produced H2, organic end-products and biomass. Organic end-products and residual glucose were removed during subsequent photofermentation by *R. sphaeroides*, with associated growth and neutralization of pH. However, photoproduction of H2 did not occur during photofermentation of the residual liquor per se due to the presence of fixed nitrogen
compounds. Nevertheless, this two-stage approach could be applied to dispose of sugar-containing industrial wastes, H2 being used for on-site power generation.

Keywords: Biohydrogen; *Rhodobacter sphaeroides*; *Escherichia coli*; Organic acids; Cross-feeding

1. Introduction

Biohydrogen is anticipated to play an important role in the future hydrogen economy, as it can be produced from readily available renewable substrates. Sugars are promising substrates for biological H2 production, being readily and renewably available and potentially giving a high yield of H2.

C6H12O6 + 6H2O → 12H2 + 6CO2. [1,2] (1)

The stoichiometric yield of 12 mol H2 per mol hexose represents the ultimate target for biohydrogen and it was suggested previously that a yield of 8 mol/mol is sufficient for economic application [3], although the economic calculation does not take into account the escalating cost of waste disposal via landfilling. No single organism is capable of performing the conversion with this efficiency. In fact the theoretical maximum yield for fermentation is 4 mol/mol as illustrated in Eq. (2) [2]. It is predicted that yields can be significantly improved by integrating fermentation and photoheterotrophy in a two stage, two-organism system.

Fermentation : $C6H12O6 + 2H2O \longrightarrow 4H_2 + 2CH3COOH + 2CO2$. (2)

Photoheterotrophy : $2CH3COOH + 4H2O \xrightarrow{h} 8H_2 + 4CO2$. [4] (3)

Eqs. (2) and (3) describe an ideal situation in which all carbon substrate is processed along the appropriate pathways and none is diverted to the formation of biomass or alternative metabolites. Practical fermentation yields tend to be around 2 mol H2 per mol hexose while yields approaching 4 mol/mol have been achieved using thermophilic fermentation [5]. Reported photoheterotrophic yields exceed 80% [4].

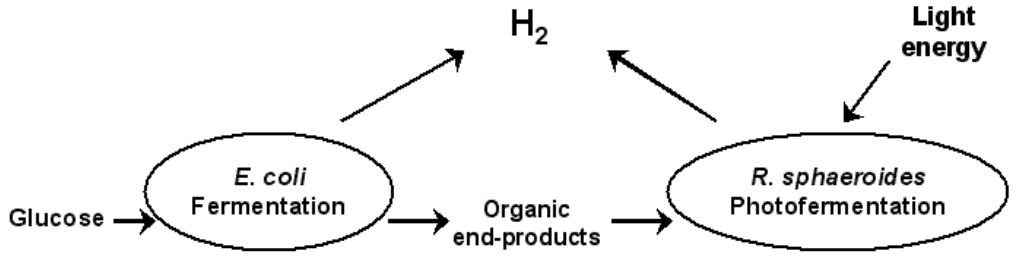

Fig. 1. (Overall scheme) In reactor 1, *E. coli* HD701 (hydrogen overproducing strain) ferments glucose to produce H2. Organic end-products (e.g. lactate, acetate, and ethanol) are cross-fed to reactor 2, where they are utilised by *R. sphaeroides* in photoproduction of H2. CO2 and biomass are formed as by-products.

Fermentation at moderate temperatures (mesophilic fermentation) is a prevalent approach, permitting biohydrogen production at a high rate and on a large scale using substrates found in organic wastes (e.g. from the food industry). This method has been extensively studied using Clostridia or extant microbial consortia from the feedstock [6–10]. Although formation of H2 during mixed-acid fermentation by *Escherichia coli* is well documented historically [11], efforts to develop the H2-producing capacity of *E. coli* have only recently achieved prominence. Unlike many other fermentative bacteria the Enterobacteria produce hydrogen by the action of the formate hydrogen lyase (FHL) system which cleaves formate to H2 and CO2 under pH stress [12]:

HCOOH –FHL→ H2 + CO2. (4)

E. coli represents a convenient vector for metabolic engineering and significantly increased rates of H2 production have been achieved through derepression of the FHL system [13–15]. The H2 yield from mesophilic fermentation is thermodynamically limited to 4 mol H2 per mol hexose [2], while experimental yields tend to be in the region of 2 mol/mol [16–18].
The accumulation of fermentation end-products (e.g. ethanol and organic acids) and fall in pH can exert sufficient 'stress' to halt mesophilic H2 production in an excess of substrate. Furthermore, the presence of organic acids in the residual medium presents a disposal issue. Utilization of fermentation end-products (for further H2 production) in a second stage would increase the economic potential of an H2-producing process by improvement of the H2 yield and reduction of the organic content of the final waste.
Anoxygenic photosynthetic bacteria (e.g. *Rhodobacter* spp.) can assimilate organic acids and produce H2 under photoheterotrophic conditions (light, anaerobiosis, high C/N ratio). Experimental yields for H2 production

from organic acids by *Rhodobacter* spp. can approach 100% [4,19]. H2 is produced as a by-product of the nitrogenase system, which functions in the fixation of N2 as ammonia, to provide a growth advantage under nitrogen limitation. Due to the high metabolic cost of maintaining active nitrogenase, its synthesis and activity are very tightly regulated with respect to the nitrogen status of the cell. The reversible inhibition of nitrogenase activity ('nitrogenase switch-off') occurs in response to micromolar concentrations of ammonium ion. However, nitrogenase is not regulated in response to the availability of its substrate (N2), and H2 production is normally performed under an argon atmosphere in order to eliminate product inhibition. In the absence of N2, ammonia is not formed but H2 production continues, as shown in Eqs. (5) and (6) [20,21]:

In the presence of N2:
$N2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH3 + H_2 + 16ADP$. (5)

In the absence of N2 (e.g. under argon):
$2e^- + 2H^+ + 4ATP \rightarrow H2 + 4ADP$. (6)

It may be practical to integrate mesophilic fermentation and photosynthesis in order to produce hydrogen from sugars with high efficiency. The use of co-cultures was reviewed in [19]. This approach is limited by the difficulty of maintaining optimum conditions for both modes of metabolism in a single reactor. It is considered preferable to operate a two-stage system: a separate fermenter and photobioreactor with cross-feeding of fermentation end-products (Fig. 1).
Kataoka et al. [16] predicted that a two-stage system for co-operative hydrogen production by *Clostridium butyricum* and photosynthetic bacteria could achieve an overall yield of 5.6 mol H2 per mol glucose. Kim et al. [17] performed a two-stage reaction with *C. butyricum* and *R. sphaeroides* and achieved an overall conversion of 1.64 mol/mol. The photosynthetic step operated at approximately 7% substrate conversion efficiency and contributed only 22% of the H2 yield. Yokoi et al. [18] reported an overall yield of 6.6 mol/mol using a twostage system in which the supernatant from a *C. butyricum/ Enterobacter enterogenes* co-culture was fed to *R. sphaeroides* M-19.
The present study represents the first instance of a two-stage system combining *E. coli* and *R. sphaeroides* in H2 production and remediation of the primary fermentation effluent. The chosen *E. coli* strain is the metabolically engineered, H2-overproducing HD701 [13] and the chosen *R. sphaeroides* strain is O.U.001 which has been studied extensively as a H2-producer [22–27].

2. Experimental

2.1. Micro-organisms and culture conditions
The H2-overproducing strain *E. coli* HD701 was kindly provided by Professor A. Böck (Lehrstuhl für Mikrobiologie, Munich, Germany) and cultured aerobically on nutrient broth (Oxoid) (30 ◦C, 200 rpm). *Rhodobacter sphaeroides* O.U.001 (DSMZ 5864) was held in stock at −80 ◦C (in 15% glycerol v/v), revived on nutrient agar (30 ◦C) and cultured anaerobically under fluorescent illumination (39.5 uE/m2/s measured with a PAR light meter SKP200, Skye Instruments Ltd.) at 30 ◦C using the SyA medium described by Hoekema et al. [28].

2.2. Determination of biomass concentration
The optical density (*E. coli* OD600, *R. sphaeroides* OD660) was measured using an UltraspecIII variable wavelength spectrophotometer (1 cm light path). OD values were used to calculate the biomass concentration (g dry biomass/l) with reference to a dried standard, prepared in triplicate by recording optical densities from dense cultures after various dilutions in deionised water. Cultures were washed twice by centrifugation and resuspension (2400 × *g*, 20 min, 4 ◦C, 50 ml deionised H2O) before drying at 60 ◦C and weighing to constant mass.

2.3. E. coli glucose fermentation
E. coli glucose fermentation was performed in a 6 l vessel (Electrolab, UK). The initial reactor contents were 2.5 l *E. coli* culture in nutrient broth (Oxoid), 2.5 l phosphate-buffered saline (1.43 g Na2HPO4, 0.2gKH2PO4, 1.0 g NaCl, 0.2 g KCl per l) and 0.55 l 1M glucose. The initial culture pH was 6.80. Anaerobiosis was established by sparging with argon (1 h), after which off-gas was collected over a solution of 1M NaOH. The temperature was maintained at 30.0 ◦C and the culture was stirred continuously (300 rpm).

2.4. Preparation of liquor
Fermentation proceeded for 24 h, after which the residual medium was treated by centrifugation (4435 × *g*, 20◦C, 10 min), and ultrafiltration of the supernatant (Acrodisc 32 mm; 0.2 μm supor membrane). The liquor was diluted 1 in 2 with sterile deionised water before inoculating with *R. sphaeroides*.

2.5. Photofermentation of R. sphaeroides on liquor
R. sphaeroides was harvested from the late exponential phase of growth *(*OD660 = 1.04*)* by centrifugation (4435 × *g*, 20◦C, 10 min). A cell concentrate containing 4 g dry biomass/l was prepared by resuspending the pellet in a sterile solution of 50 mg/l MgSO4 · 7H2O and 25 mg/l CaCl2 with micronutrients as described by Hoekema et al. [28]. This suspension (5 ml) was inoculated

into 195 ml pre-degassed liquor to give an initial biomass concentration of 0.2 g/l. The culture was sealed using butyl rubber stoppers with aluminium crimp seals and sparged with argon (20 min) to remove N2 and to establish anaerobiosis. Light was provided by two fluorescent 10W lamps (Eterna). The intensity of photosynthetically active radiation (measured as 2.1) was 10 µE/m2/s (47W/m2). Reactors were stirred magnetically. The temperature was maintained at 30 °C using a water bath.

2.6. Measurement of hydrogen production

Exit gas from *R. sphaeroides* reactors was directed though a bubbler, which contained a solution of 200 g/l NaCl (pH 2) and then into a graduated glass tube containing 1M NaOH. The bubbler functioned to maintain anaerobiosis and the NaOH served to remove CO2 from the exit gas so its displacement from the tube would indicate the volume of H2 produced.

2.7. Chemical analyses

5ml samples were withdrawn at intervals from 200 ml *R. sphaeroides* cultures. OD660 and pH were measured immediately before cells were separated by centrifugation (13000 × *g*, 20◦C, 4 min). Supernatants were stored at −20 ◦C before analysis. Organic acids were measured by anion HPLC (Table 1); glucose was assayed using the colorimetric dinitrosalicylic acid assay [29]; ammonia was determined using the colorimetric Nessler assay [30]; protein was measured using the colorimetric bicinchoninic acid assay (Sigma procedure TPR0562) and ethanol was determined colorimetrically by monitoring the enzymatic reduction of NAD using alcohol dehydrogenase (Sigma A-6338, assay concentration: 2.64 U/ml) after pre-removal of aldehyde by aldehyde dehydrogenase (Sigma A-7011, assay concentration: 16.18 U/l).

Table 1. Conditions for anion chromatography

Columns	IonPac AS11-HC Analytical (4mm) IonPac AG11-HC Analytical (4mm)				
Eluent					
	A Deionised water (18.2 MΩ-cm)				
	B 5 mM NaOH				
	C 100 mM NaOH				
	D 250 mM NaOH				
Gradient (%)	Time (min)	A	B	C	D
	-2	60	40	0	0
	0 (inj.)	60	40	0	0
	1	88	12	0	0
	10	88	12	0	0
	20	80	0	20	0
	23	20	0	0	80
	24	20	0	0	80
	24.01	60	40	0	0
	27	60	40	0	0
Flow rate	15 ml/min				
Inj. volume	15 µl				
Detection	Suppressed conductivity, ASRS Autosuppression recycle mode				

3. Results and discussion

3.1. First stage fermentation by E. coli and second stage photofermentation

The initial fermentation using *E. coli* (see materials and methods) was described in detail previously [31]. In this 24 h batch fermentation, H2 was produced at a rate of 52.0 ml/l/h and at a molar yield of 0.376. Using an improved methodology (pH controlled at 5.5, fed-batch, washed cells pre-grown in the presence of 5 g/l sodium formate) the H2 yield was improved to 2.4 mol/mol (M.D. Redwood, D.W. Penfold and L.E. Macaskie unpublished). Liquor from the 24 h batch fermentation was used to test the remediation-potential of *R. sphaeroides* via photofermentation. The cell-free liquor was inoculated with *R. sphaeroides* and monitored for growth (Fig. 2A), neutralization of pH (Fig. 2B), removal of organic components (Fig. 3) and H2 production.

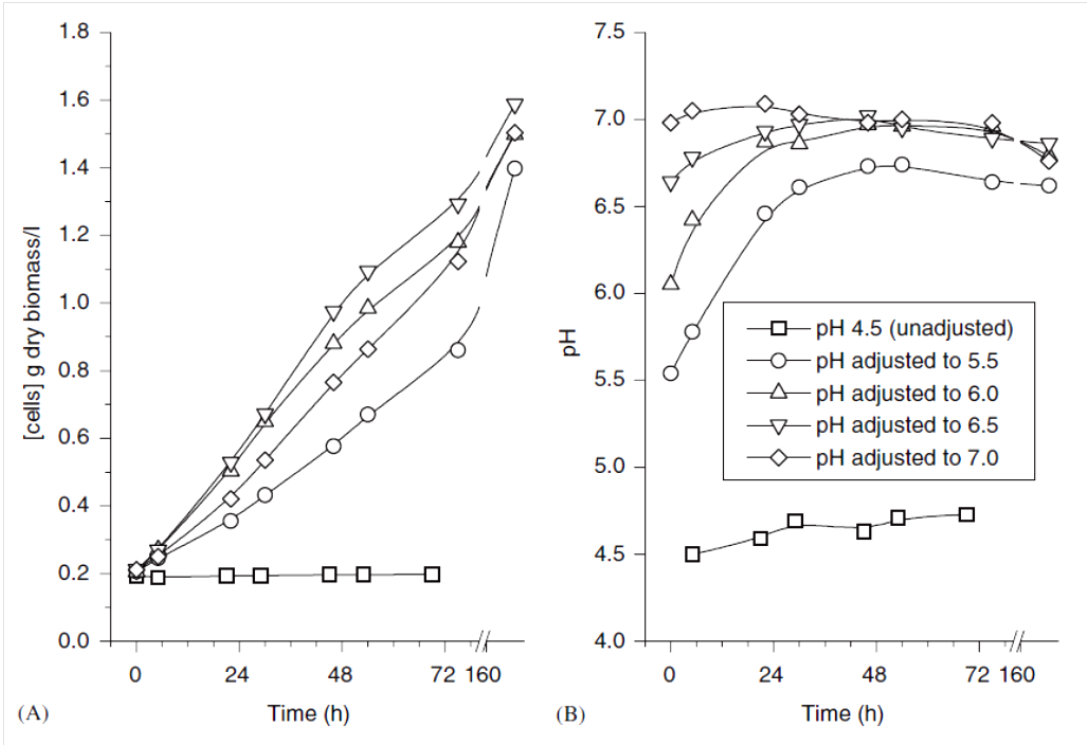

Fig. 2. Growth of *R. sphaeroides* on *E. coli* spent fermentation liquor and neutralization of pH: (A) Growth of *R. sphaeroides* on liquor from *E. coli* fermentation (Table 2) adjusted to different pH values prior to inoculation and (B) pH profile during growth at different initial pH values.

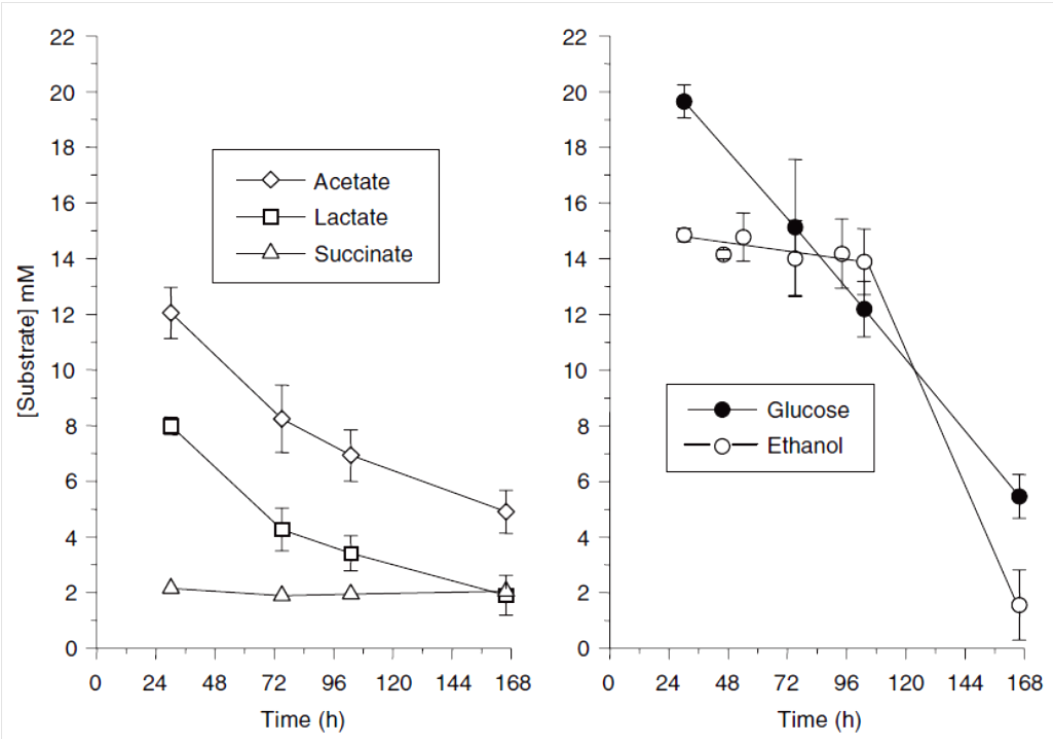

Fig. 3. Removal of organic compounds from fermentation liquor by *R. sphaeroides*. Data represent means from at least three replicate experiments. Bars represent standard error of the mean.

3.2. Cultivation of R. sphaeroides on E. coli fermentation liquor

Fermentation by *E. coli* resulted in acidification of the medium from pH 7.0–4.5 due to the formation of organic acids (Table 2). As shown in Fig. 2 spent liquor at pH 4.5 did not permit growth of *R. sphaeroides*. However, when the pH was adjusted to 5.5, growth and further neutralization of pH occurred rapidly (growth rate: 0.20 g/l/day). The highest growth rate (0.41 g/l/day) was observed after the pH was adjusted to 6.5. These observations were in agreement with previous reports [25].

While the required pre-adjustment of pH was only 1 pH unit, this would introduce an additional cost in largescale cultivation of photoheterotrophic bacteria. Further work would be required to achieve acid-tolerant remediation of *E. coli* fermentation liquor.

3.3. Removal of organic components from E. coli fermentation liquor

The composition of typical *E. coli* spent fermentation liquor is shown in Table 2. The low turbidity, high organic content and high nitrogenous content make this a suitable substrate for cultivation of *R. sphaeroides*. As shown in Fig. 3 the primary carbon sources for growth were glucose, acetate and lactate, which were removed at average rates of 0.46, 0.30, and 0.17 mmol/day/reactor, respectively. Although succinate is usually a preferred carbon source for growth of purple non-sulphur bacteria [32], it was not consumed in these experiments, possibly due to its low initial concentration.

The consumption of ethanol was secondary to the other substrates, commencing only after ~100 h. By combining the removal rates for glucose, acetate and lactate a total removal rate of 3.87 mmol C/day/reactor was calculated.

These results show that the two-stage system described here could be applied for remediation of industrial

Table 2: Properties of fermentation liquor.

Fermentation properties		General properties	
Glucose	40 mM	pH	4.5
Ethanol	20 mM	Turbidity	Low
Total organic acids	38 mM	Ammonia	4.4 mM
Acetate	20 mM	Protein	2.30 g/l
Lactate	15 mM	Chloride	46 mM
Succinate	3 mM	Phosphate	5.2 mM

[α] Liquor was diluted 1 in 2 before inoculating with *R. sphaeroides*.

sugary wastes. Suitable organic feedstocks for *E. coli* can be obtained at low cost, also helping to forestall landfill as a disposal issue [13,31]. Out-flow from the photobioreactor was recycled back into the primary *E. coli* fermenter showing that metabolic products of *R. sphaeroides* are not inhibitory to further H2 production by *E. coli* since a second burst of H2 evolution was observed (M.D. Redwood, D.W. Penfold and L.E. Macaskie, unpublished).

3.4. Potential for H2 production from E. coli fermentation liquor

Theoretically, the organic acids present in fermentation liquor could be converted to H2 to yield 0.19 mol (4.83 l) H2 per litre fermentation waste (excluding that from ethanol and glucose). Although *R. sphaeroides* assimilated organic acids from the fermentation liquor (Fig. 3), H2 formation was not observed. This was attributed to the presence of fixed nitrogen sources (4.4mM ammonium ion, 2.3 g/l protein: Table 2).

A positive control was performed using a synthetic medium lacking nitrogenous components, but containing glucose, ethanol and organic acids at concentrations identical to those in the *E. coli* spent liquor. H2 was produced at a rate of 124 ml H2/l culture/day (10.5 ml/h/g dry biomass).

Repressive concentrations of ammonium ion were present in the liquor (together with significant protein: Table 2) and the rate of removal (38 μmol NH_4^+ /l/day) was insufficient to overcome repression of nitrogenase activity. Nitrogenase fixes N2 gas as ammonia to overcome nitrogen-limitation, forming H2 as a by-product. This metabolically demanding process is repressed in the presence of fixed nitrogen (see earlier). The repressibility of nitrogenase is a common bottleneck in photobiological H2 production, which may be overcome by the use of various strategies, for example:

- Denitrification of the fermentation liquor could be achieved by alkalinisation followed by boiling. This method was used to prepare food processing wastewater and sewage sludge for use as fermentation feed [17]. Alternatively, classical deionisation techniques could potentially remove NH_4^+ with some selectivity, but either approach would add significant process costs.
- A two-part photobioreactor permitting ammonia consumption for growth in the first reactor followed by H2 production in a nitrogen-limited second reactor [33]. Wild-type bacterial strains could be employed in this approach, but process costs would be increased as compared to those for a single reactor arrangement.
- The use of nitrogenase-derepressed mutants which exhibit nitrogenase expression in the presence of

fixed nitrogen [34,35]. Although industry is often reluctant to accept engineered strains, this approach could offer the efficient photoproduction of H2 from a nitrogenous feed in a single stage.

• A membrane separation system could be used to separate the organic acids. Preliminary tests using this approach have suggested that this permits H2-evolution in the *R. sphaeroides* reactor receiving organic acids from the *E. coli* primary fermentation (M.D. Redwood and L.E. Macaskie, unpublished). Elucidation of mass balances and consideration of process economics are in progress.

In conclusion, there is significant potential for H2 production from fermentation liquor. It should be noted that the *R. sphaeroides* photofermentation was not performed under optimum conditions at this stage. This was suggested by a relatively low rate of H2 production observed for the positive control. This may be attributed to the use of fluorescent light, whereas tungsten/halogen lamps are preferable due to the emission of wavelengths in the range of 800 and 850 nm, corresponding to the absorption maxima of bacteriochlorophylls [4]. The use of an improved light source (tungsten; 250 μ/m2/s) could permit higher rates of substrate removal than those reported here, but the high nitrogen content would still prevent photoproduction of H2.

4. Conclusions

R. sphaeroides was able to grow on fermentation liquor (after pH adjustment), simultaneously removing organic components and neutralizing the pH. Nitrogenase activity (H2 production) was inhibited by the high nitrogen content of the liquor but was shown in nitrogen-free medium. This data highlights that inhibition of nitrogenase is the foremost challenge stalling the development of a two-stage system for biohydrogen, but numerous strategies have the potential to overcome this. Future work will aim to achieve photoproduction of H2 from fermentation liquor by the use of membraneseparation technology.

Acknowledgements

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